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RESEARCH PAPER

Effects of long-term elevated CO_2 on N_2-fixing, denitrifying and nitrifying enzyme activities in forest soils under *Pinus sylvestriformis* in Changbai Mountain

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Abstract: A study was conducted to determine the effects of elevated CO_2 on soil N process at Changbai Mountain in Jilin Province, northeastern China (42º24´N, 128º06´E, and 738 m elevation). A randomized complete block design of ambient and elevated CO_2 was established in an open-top chamber facility in the spring of 1999. Changpai Scotch pine (*Pinus sylvestris* var. *sylvestriformis* seeds were sowed in May, 1999 and CO_2 fumigation treatments began after seeds germination. In each year, the exposure started at the end of April and stopped at the end of October. Soil samples were collected in June and August 2006 and in June 2007, and soil nitrifying, denitrifying and N_2-fixing enzyme activities were measured. Results show that soil nitrifying enzyme activities (NEA) in the 5–10 cm soil layer were significantly increased at elevated CO_2 by 30.3% in June 2006, by 30.9% in August 2006 and by 11.3% in June 2007. Soil denitrifying enzyme activities (DEA) were significantly decreased by elevated CO_2 treatment in June 2006 ($P < 0.012$) and August 2006 ($P < 0.005$) samplings in our study; no significant difference was detected in June 2007, and no significant changes in N_2-fixing enzyme activity were found. This study suggests that elevated CO_2 can alter soil nitrifying enzyme and denitrifying enzyme activities.

Keywords: elevated CO_2; forest soil; nitrifying enzyme; denitrifying enzyme; N_2-fixing enzyme

Introduction

Due to combustion of fossil fuel, deforestation and intense agriculture, the concentration of atmospheric CO_2 increased from 280 μmol·mol^{-1} at the beginning of industrialization to the present 365 μmol·mol^{-1} and would continue to be rising by about 1% per year. According to some models and experiments, terrestrial ecosystems can sequester a part of the additional carbon fast enough to help to counteract CO_2 emissions. Numerous studies conducted with elevated CO_2 concentrations (Catovsky and Bazzaz 1999; Norby et al. 1999; Norby et al. 2000; Oren et al. 2001), have showed a greater biomass gain of plants, higher fine root and leaf litter C/N in some species (Cotrufo and Ineson 1995). However, plant aboveground carbon accumulation may be limited by nutrients, particularly nitrogen (Townsend et al. 1996; Nadelhoffer et al. 1999). Recently some studies showed that when CO_2 enrichment increases soil C:N, decomposing microorganisms require more N. This effect could decrease N mineralization, the major source of nitrogen for plant growth (Gill et al. 2002). Thus, the interactions between C and N might influence the terrestrial ecosystem responses to elevated atmospheric CO_2 concentrations, which are great importance to atmosphere-biosphere interactions, and thus to global climate. Unfortunately, the impact of the rising CO_2 on the N cycle of terrestrial ecosystems is still unclear, and it is also not clear how the biological processes driving soil N availability would be altered by the CO_2 enrichment, e.g. nitrification, denitrification and N-fixing (Barnard et al. 2004).

Soil nitrification and denitrification are the microbial processes, which are responsible for the transformation of N into forms that are easily utilized by plant or lost from rhizosphere, and their rates could be modified by the rising CO_2 because the processes in the soil are likely to be sensitive to these CO_2-induced changes in soil labile C, soil water content and litter quality (Barnard et al. 2005a). However, the results of previous studies are quite contradictory (Barnard and Leadley 2005), as the rates of soil nitrification and denitrification have been observed to increase (Carnol et al. 2002; Phillips et al. 2001), decrease (Barnard et al. 2005b; Barnard et al. 2004) or remain

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stable (Hungate et al. 1997; Barnard et al. 2006) under elevated atmospheric CO_2. For example, Barnard et al. (2004) indicated that nitrification activity strongly decreased at elevated CO_2 in the Holcus mesocosms, but was unaffected in Festuca systems, and CO_2 treatment had an only smaller increase in denitrification enzyme activity. Additional N is required to sustain the enhanced plant growth under elevated CO_2 and promote C sequestration for the long term (Hungate et al. 2003), but the source of this additional N supply has not been identified. One potential source of increasing plant available N is thought to increased heterotrophic N_2 fixation in the soil. Gifford et al. (1996) reported that the increased substrate availability under elevated CO_2 could increase N_2 fixation. However, Hofmockel and Schlesinger (2007) did not detected CO_2 effect on potential nitrogenase activity in Duke FACE soil.

The objective of the present study was to assess the effect of elevated CO_2 on soil nitrification, denitrification and N_2-fixing enzyme activities in a long-term in situ CO_2 enrichment experiment in a temperate forest volcanic soil. In the 8th and 9th year of this experiment, soil was sampled and the enzyme activities were measured.

Materials and methods

Experimental site, design and sampling

The experimental fields were located at Changbai Mountain in Jilin Province, northeastern China (42º24´N, 128º06´E, and 738 m elevation). The soil is a dark-brown soil developed from volcano ashes. The topography is basaltic mesa, and the parent rock is loose volcano ash sand. The ecosystem is temperate with a mean annual temperature of 5ºC and annual average precipitation of 967.3–1400 mm. A randomized complete block design of ambient and elevated CO_2 was established in an open-top chamber facility at the research station of Changbai Mountain Forest Ecosystems of Chinese Academy of Sciences in the spring of 1999. Open-top chambers (each 4.2 m in diameter with hexagon and 4 m in height enclosed with a clear glass open-top chamber) were utilized to control CO_2 levels. Changpai Scotch pine (*Pinus sylvestris* var. *sylvestriformis* (Takenouchi) Cheng et C. D. Chou) seeds were prepared and sowed in May, 1999. CO_2 fumigation treatments began after seeds germination in May 1999. In each year, the exposure started at the end of April and stopped at the end of October (the whole growing season). Half of the chambers were maintained at ambient atmospheric CO_2 concentrations (ca. 350 μmol·mol^{-1}); others were maintained at elevated levels (ca. 500 μmol·mol^{-1}) by dispensing 100% CO_2 into the blower fans only in the daytime. Elevated CO_2 concentrations were maintained by continuously monitoring CO_2 concentrations in elevated and ambient-level chambers with an infrared gas analyzer (A-SENSE-D, SenseAir, Sweden) by a computer control system that recorded 10-second averages of CO_2 concentration every 3 min and then periodically adjusting the flow of 100% CO_2 into the chambers.

Soil samples were collected three times: June, August 2006 and June 2007. At each sampling date, 8–12 soil cores (3 cm in diameter and 5–10 cm at deep) were collected within each chamber. The samples were homogenized by passing soil through a 2-mm sieve. The subsamples were analyzed within 24 h after sampling. The other subsamples were air-dried for measuring of soil pH and soil organic carbon.

Nitrifying, denitrifying and N_2-fixing enzyme activity measurements

Subsamples of soil were dried at 105ºC for 12 h to determine gravimetric water content. Denitrification enzyme activity (DEA) was measured using the acetylene-based anaerobic assay as described by Smith and Tiedje (1979). Briefly, ten grams equivalent dry soil were placed into 150 mL plasma flasks, and 6 mL distilled water containing KNO_3, glucose, and glutamic acid was added to achieve a final concentration of 200 μg N-NO_3^-·g^{-1} dry soil and 1 mg C·g^{-1} dry soil. The atmosphere of each flask was replaced by a 90:10 N_2–C_2H_2 mixture to provide anaerobic conditions and inhibition of N_2O-reductase activity. The N_2O efflux was measured in the flask after four hours. N_2O concentrations were immediately analyzed on a gas chromatograph equipped with an electron capture detector (GC HEWLETT 5890 PACKARD SERIES Ⅱ). DEA was expressed as μgN·h^{-1}·g^{-1} dry soil.

Nitrificaiton enzyme activity was measured according to Lensi et al. (1986). From each soil sample, six subsamples of 10 g equivalent dry soil were placed in 150 ml plasma flasks. Three subsamples were used to estimate the initial NO_3^-content. These subsamples were supplied with 8 mL of a suspension of a denitrifying organism (*Pseudomonas fluorescens*, OD_{580} = 2) in a solution containing glucose and glutamic acid (final soil C content for each: 0.5 mg C·g^{-1} dry soil). The flasks were sealed with rubber stoppers and the atmosphere of each flask was replaced by a N_2-C_2H_2 mixture (90-10). The three other subsamples were used to determine NO_3^- accumulation: they were enriched with 2 mL of an $(NH_4)_2SO_4$ solution (final soil N concentration 0.2 mg·g^{-1} dry soil) in order to ensure a moisture content equivalent to 80% water-holding capacity and no limitation by ammonium (the presence of also limits assimilation by micro-organisms). Flasks were then sealed with parafilm, which prevents soil from drying but allows gas exchange, and incubated at 27ºC for 48 h in a horizontal position to ensure optimal, homogeneous aeration of the soil. After the aerobic incubation which allows nitrate to accumulate, the soil samples were enriched with 4 mL of a *P. fluorescens* suspension (OD_{580} = 2) in a solution containing glucose and glutamic acid (concentrations as above). Then anaerobiosis and N_2O inhibition were obtained in the flasks as described above and the N_2O accumulation was surveyed until a constant value was reached. N_2O was analyzed on a HEWLETT 5890 gas chromatograph. The enzymatic potential of nitrification was computed by subtracting the nitrate initially present in the soil from the nitrate accumulated after aerobic incubation and expressed as μg NO_3–N produced by per gram of dry soil and per hour.

Nitrogenase activity was determined using the acetylene (C_2H_2) reduction technique (Turner and Gibson 1980). Fresh soil

(equivalent to 10 g oven dried) was incubated in a 150-mL sterile flask with a rubber stopper. Soils were amended with a solution containing glucose (4 mL, to make 1 mg C/g dry soil) and disodium malate (1 mg C/g dry soil). The gas atmosphere in the flashes was replaced with a 90:10 mixture of air:acetylene and the flasks were incubated for three days at 27ºC. Gas (500 mL) was sampled daily and C_2H_4 concentration determined using gas chromatography with a flame ionization detector (GC HEWLETT 5890 PACKARD SERIES Ⅱ). Nitrogenase activity was determined from the kinetics observed between 24 and 48 h of incubation. N_2-fixation was calculated using a conversion factor of 1/3 N_2 reduced per C_2H_2 reduced (Burris 1974).

Statistical analysis

Data were analyzed using one-way ANOVAs for each individual date with treatments as the factor. ANOVA analyses were performed with SPSS 13.0 statistical software package (SPSS Inc.).

Results and discussion

The pH values were 5.77±0.01 and 5.49±0.02 for ambient and elevated CO_2, respectively. Thus, elevated CO_2 significantly increased soil acidity. Soil organic C content averaged 82.20±1.31g C/kg soil and 82.73±1.91g C/kg soil under ambient and elevated CO_2, respectively. Additionally, soil bulk density was decreased with elevated CO_2 treatment, but no significant difference was found. Nitrifying enzyme activity (NEA) was determined in the soils under elevated and ambient CO_2 is provided in Fig. 1 and Table 1. NEA activity was significantly increased across all three samplings from elevated CO_2 compared to ambient treatment ($P < 0.01$). Soil NEA activity in the 5–10 cm soil layer was significantly increased at elevated CO_2 by 30.3% in June 2006, by 30.9% August 2006 and by 11.3% in June 2007. Nitrification, which is performed by ammonia-oxidizing bacteria converting ammonium (NH_4^+) to nitrite (NO_2^-) and then by nitrite-oxidizing bacteria converting the latter to nitrate (NO_3^-), is directly involved in plant N nutrient supplies and soil N losses through leaching. NEA is generally favored in well-aerated soils, at high NH_4^+ availability. The below-ground effects of CO_2 are mediated by changes in plant C allocation (Norby 1994) and the quantity and quality of leaf litter and root exudates (Norby et al. 1994; 2001). Nitrification is aerobic, so that indirect effects of the changes in higher fine root growth and turnover and soil bulk density under elevated atmospheric CO_2 on soil O_2 concentration may play a key role in controlling the process. In our study plots, the increases in plant biomass were measured continuously through the fumigation years, which could potentially increase ammonium availability in the soil through an increase of root-derived carbon. Additionally, the soil bulk density was also found to decrease under elevated CO_2 condition, which can enhance soil O_2 concentration. Our results are agreed with the findings of Lata et al. (2000), who found a strong positively correlation between nitrification and root biomass. The increase in nitrifying enzyme activity was also consistent with the lower value of soil pH with elevated CO_2.

Ammonium, the initial substrate for nitrification, which is tightly retained in ecosystems, difficultly uptake by plant, nitrate is easy uptake by plant and also easily lost through leaching. Elevated CO_2 could potentially alter nitrification through modifications of NH_4^+ availability because it has been shown to increase gross mineralization in a number of studies (Zak et al. 2000; Barnard et al. 2004b). Thus the increase of NEA under elevated CO_2 would be beneficial to N uptake of plant, also may be result in N leaching enhancement and render N lost from ecosystem. Elevated CO_2 could also stimulate plant N uptake (Hu et al. 2001) and soil biological N fixation (Hungate et al. 1999), further stimulate mineralization of ammonium for microbial nitrification. All of these results suggest that the rising CO_2 concentrations may significantly increase soil nitrification potential through altering soil chemical properties, e.g. soil pH, soil bulk density, etc..

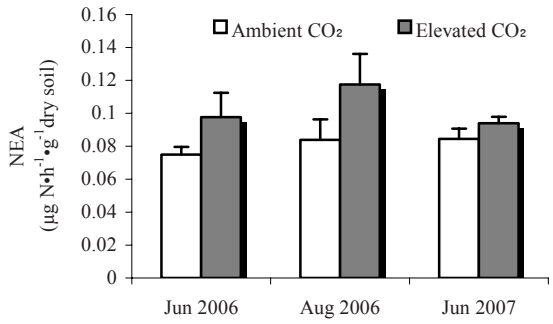

Fig. 1 Nitrifying enzyme activity in *P. sylvestriformis* 5–10 cm soil in June 2006, August 2006 and June 2007. Values represent the mean and standard error of ambient and elevated CO_2 treatments at each open top chamber.

Table 1. *P*-values from Student's t-test showing the effects of elevated CO_2 on soil N_2-fixing, denitrifying and nitrifying enzyme

	June 2006		August 2006		June 2007	
	t	*P*	t	*P*	t	*P*
N_2-fixing enzyme	1.551	0.248	5.200	0.072	3.288	0.167
Denitrifying enzyme	12.860	0.012	19.006	0.005	1.555	0.236
Nitrifying enzyme	10.841	0.010	11.175	0.010	8.78546	0.018

DEA activity was significantly decreased by elevated CO_2 treatment in June 2006 ($P < 0.012$) and August 2006 ($P < 0.005$) samplings in our study; no significant difference was detected in June 2007 (Fig. 2, Table 1). Both O_2 restriction and presence of NO_3^- are necessary for appreciable denitrification. Elevated CO_2 is often reported to tend to reduce stomatal conductance of plants which results in higher water use efficiency and higher soil water content (Körner 2000), which favor DEA of soil. Several studies did have observed enhanced N_2O emission and/or denitrification potential from elevated CO_2 treated soil (Barnard et al. 2005). However, our study detected decreases in denitrifying enzyme activity. One possible explanation is that increased root growth could cause higher supply of oxygen (Kang et al. 2001), which

can strongly inhibit denitrifier bacteria. The supply increase of oxygen can also be obtained from decrease in soil bulk density with elevated CO_2. In a review of the potential effects of global change on denitrification, Barnard et al. (2005) suggested that the CO_2 enhancement was generally associated with a decrease in soil nitrate, which might reduce the availability of electron acceptors for denitrification, thus reducing its activity. Studies on denitrifying enzyme activity indicated that slightly alkaline conditions favor denitrification (Valera and Alexander 1961), whereas low pH conditions generally inhibited the process (Weier and Gilliam 1986; Christensen and Tiedje 1988; Christensen et al. 1990). Similar results have been reported by Deiglmayr et al. (2004), who found a decreased nitrate reductase activity in the rhizosphere of *Lolium perenne* and *Trifolium repens* under elevated CO_2.

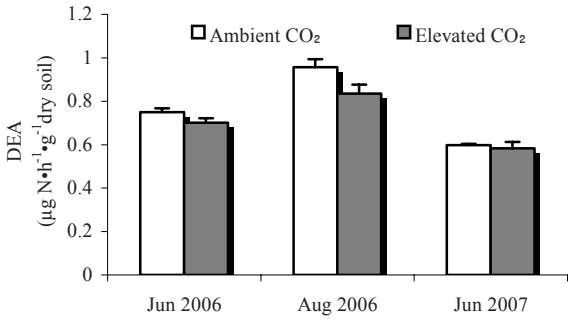

Fig. 2 Denitrifying enzyme activity in *P. sylvestriformis* 5–10 cm soil in June 2006, August 2006 and June 2007. Values represent the mean and standard error of ambient and elevated CO_2 treatments at each open top chamber.

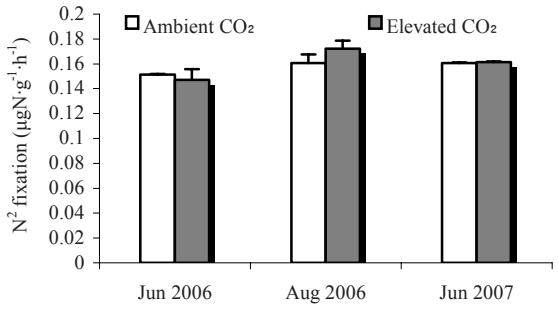

Fig. 3 N_2-fixing enzyme activity in *P. sylvestriformis* 5–10 cm soil in June 2006, August 2006 and June 2007. Values represent the mean and standard error of ambient and elevated CO_2 treatments at each open top chamber.

Elevated CO_2 can have a positive effect (Dakora and Drake 2000; Cheng et al. 2001; Hoque et al. 2001) or no effect (Hofmockel and Schlesinger 2006) on soil N_2-fixing enzyme activity. We found only a marginally significant difference in soil N_2-fixing enzyme activity response to elevated CO_2 in August 2006 ($P<0.072$). There are several possible explanations for this lack of responsiveness. Soil water content may be a key factor in the response of soil N_2-fixing enzyme to elevated CO_2 (Hofmockel and Schlesinger 2006). In laboratory manipulations of intact soil cores, Hofmockel and Schlesinger (2006) found that water additions could increase N_2 fixation 25-fold. Soil water limits diffusion of O_2 in soils, which can react with the Fe element of the nitrogenase enzyme, rendering the enzyme permanently inactive (Stacey et al. 1992). In our study, elevated CO_2 had no significant effect on soil water content. The bacteria that primarily responsible for N_2-fixation under anaerobic conditions, for example the members of *Clostridium* and *Bacillus*, always believed to be independent on soil pH (Jurgensen and Davey 1970). Although laboratory experiments indicate that the growth of pure cultures of N_2-fixers, such as *Azospirillzim lipofenmi* (Day and Döbereiner 1976) is pH-dependent, the field survey by Garcia et al (1974) strongly suggests that pH effect would be less marked than expected. Thus the results proposed that the increase of soil acidity under elevated CO_2 is apparently insufficient to modify soil heterotrophic N_2-fixing enzyme activity. The availability of organic substrates could limit soil heterotrophic N_2 fixation rates, because the N_2 fixation is energetically expensive process. Hofmockel and Schlesinger (2006) suggested that a greater supply of C derived from higher net primary productivity under the increasing CO_2 could stimulate soil heterotrophic N_2 fixation. In our study, soil N_2-fixing enzyme activity under elevated CO_2 increased by 7.2% than ambient CO_2 in August 2006, which is high photosynthesis period and rainy season in Changbai mountain region (Zhou 2003). These results are consistent with the results of Cheng (2001) who found soil heterotrophic N_2 fixation was increased under elevated CO_2 in the environments with low redox potential. Our results indicated that the stimulation of elevated CO_2 on soil heterotrophic N_2-fixing potential was dependent on above-ground plant and/or soil water variation.

Conclusion

Our results, together with other studies, suggest that elevated CO_2 can alter soil nitrifying enzyme and denitrifying enzyme activities through altering soil chemical properties, whereas soil N_2-fixing enzyme may be insensitive to the CO_2-induced changes in soil pH, soil bulk density and soil organic mater quantity in forest soil developed from volcano ashes with specific types of humic substances and higher organic carbon content. Elevated CO_2 may be decrease efflux of N_2O in soil developed from volcanic ashes, increase N efficiency of plant.

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